BIOSYNTHESIS OF THE COAT PROTEIN OF COLIPHAGE f2 BY E. COLI EXTRACTS*

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Nirenberg and Matthaei[1] have discovered an assay system in which RNA serves as an activator of protein synthesis in *E. coli* extracts. RNA fractions from cells,[1] synthetic polyribonucleotides,[2, 3] and viral RNA[1, 4] can all stimulate amino acid incorporation into acid-insoluble products in *E. coli* extracts. Although in each case the product formed is presumed to be a protein or polypeptide whose structure is uniquely determined by the RNA, the products have not as yet been completely

analyzed. A recent paper by Tsugita *et al.* presents evidence suggesting that the coat protein of TMV is synthesized by *E. coli* extracts.[5] With the availability of a bacteriophage attacking *E. coli* and containing RNA (coliphage f2),[6] it became possible to use the RNA isolated from this phage to stimulate amino acid incorporation into protein and to identify at least part of the product as the coat protein of the phage.

Materials and Methods.—Phage RNA and protein: Large amounts of the phage f2 were prepared by scaling up the procedure of Loeb and Zinder.[6] RNA was prepared by deproteinization of the phage suspension by shaking with phenol and precipitation of the RNA from the aqueous phase by the addition of two volumes of ethanol. The RNA was kept at $-20°$ in distilled water and used as needed. Its concentration was determined by measuring the optical density at 260 $m\mu$ and assuming a specific absorption of 24 per mg. Phage protein was prepared by extraction of purified f2 in 67 per cent acetic acid followed by dialysis and lyophilization. The following properties of the protein are pertinent to this paper. On the basis of chromatography on DEAE-cellulose, starch gel electrophoresis, and ultracentrifugation, the protein appears to be homogeneous. It has a probable molecular weight of 20,000 and contains 10 leucine, 5 lysine, 3 arginine, and no histidine residues per molecule.[7] Digestion of the oxidized protein by trypsin gives nine ninhydrin-reacting components which are resolved by two-dimensional electrophoresis; one of these has the mobility of free lysine. The details of the digestion procedure used in these experiments will be described below.

E. coli extracts: Preparation of the *E. coli* extracts was based on the procedure described by Nirenberg and Matthaei.[1] *E. coli* B was harvested in the log phase of growth. The washed, frozen cell paste was ground with $2^1/_2$ parts of Alumina (Alcoa A-303) and was extracted with $1^1/_2$ volumes of 0.01 *M* pH 7.4 Tris buffer that was 0.01 *M* in magnesium acetate. After the addition of 5 μg/ml of DNase, the extract was centrifuged at 15,000 $\times$ *g* for 10 min. To the resulting supernatant, β-mercaptoethanol was added to a final concentration of 0.005 *M*, and then it was centrifuged at 30,000 $\times$ *g* for 30 min. The 30,000 $\times$ *g* supernatant fraction (S-30) was incubated at 35° for 30 min with the components required for amino acid incorporation into protein: 0.003 *M* ATP, 0.0002 *M* GTP, 0.01 *M* phosphoenolpyruvate, 30 μg/ml pyruvate kinase, 0.01 *M* glutathione, 0.011 *M* magnesium acetate, 0.03 *M* KCl, 0.05 *M* Tris HCl pH 7.8, 4×10^{-5} *M* of each amino acid, 5 μg/ml DNase, 1 mg/ml sRNA, and a volume of S-30 equal to half the total incubation volume. After overnight dialysis against 0.01 *M* Tris HCl pH 7.8, 0.01 *M* magnesium acetate, 0.03 *M* KCl, and 0.005 *M* β-mercaptoethanol, the preincubated mixture was stored at $-20°$.

For the incorporation experiments to be described, the components present during preincubation (minus additional sRNA and DNase) were incubated at 35° with the appropriate C^{14}-amino acids using an amount of preincubated S-30 (containing 4.6 mg of ribosomal RNA/ml) equal to a quarter of the total volume. Amino acid incorporation into proteins was determined by counting the hot acid-precipitate (5 per cent trichloroacetic acid, 90° for 15 min) after washing with TCA and ethanol-ether.

Electrophoresis: High-voltage electrophoresis[8] of tryptic digests of protein (fingerprinting) was performed on Whatman 3 MM paper in a Servonuclear Company tank under Varsol (Standard Oil Co., N.J.). The first dimension was run for 1 hr in pH 4.7 buffer containing by volume 25 ml of acetic acid and 25 ml of pyridine/liter of water. The field strength was 50 volts/cm. At the completion of the run, the paper was dried and the strip containing the peptides cut out and sewn onto another piece of paper. The second dimension was run for 3–4 hr in pH 1.9 buffer containing 87 ml of acetic acid and 25 ml of 88 per cent formic acid/liter of water. The field strength was 20 volts/cm.

Radioactive amino acids: C^{14}-leucine, -arginine, and -lysine with specific activities of 144 mC/mmole and H^3-leucine with specific activity of 5 C/mmole were obtained from New England Nuclear Corporation, Boston, Mass. C^{14} counting was done with a windowless gas flow counter. H^3 and C^{14} in the same sample were determined by counting with and without a thin window and then making the appropriate corrections.

Results.—Characteristics of the amino acid incorporating system: The following

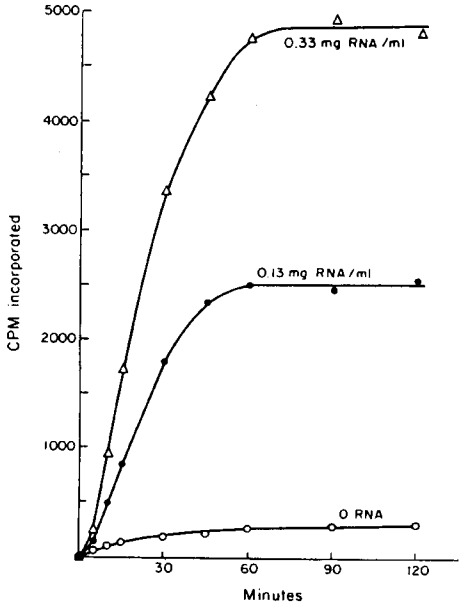

FIG. 1.—Kinetics of leucine incorporation with different concentrations of f2 RNA. The incubation mixtures were as described under *Methods* and contained C^{14}-leucine (1.5×10^7 cpm/μmole) and the concentration of RNA indicated. Each point represents the acid-insoluble counts in 0.20 ml.

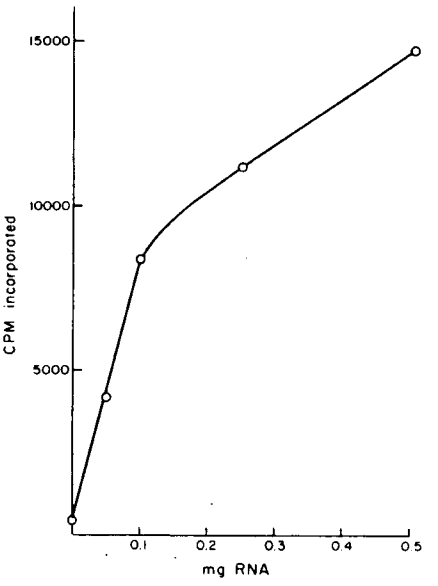

FIG. 2.—Effect of increasing concentration of f2 RNA on leucine incorporation. The components given in *Methods* were incubated with C^{14}-leucine (1.5×10^7 cpm/μmole) at 35° for 90 min. Total volume was 0.50 ml.

experiments show the extent of the stimulation of amino acid incorporation by *E. coli* extracts when exposed to f2 RNA. Figure 1 shows the kinetics of incorporation of leucine at different RNA concentrations. Both the rate of incorporation and the final amount of product vary directly with the RNA concentration. In Figure 2, the effect of increasing RNA concentration on the amount of leucine incorporated is shown. With the concentration of components used, there is a linear response up to 200 μg/ml and then a sharp break in the curve. Above this concentration, a smaller response per unit of RNA added is observed. Saturation is not achieved even at our highest RNA concentration, which stimulates some 40-fold. In the linear portion of the concentration curve, 0.53 mμ moles of leucine is incorporated per 100 μg of RNA. From this value, one can calculate that about 30 nucleotide residues are required for each molecule of amino acid incorporated. This estimation is based on the specific activity of the leucine, on the percentage composition of the leucine in the phage protein, and on the assumption that any other proteins synthesized have the same percentage of leucine as does the f2 protein.

To demonstrate that the reaction has the properties expected of a protein-synthesizing system, the antibiotics chloramphenicol and puromycin were added to the system. Both of these inhibitors of protein synthesis inhibited the incorporation of leucine (Table 1).

Identification of the product: Two kinds of experiments are to be described by which we characterize the product. The first involves chromatography of the

TABLE 1

EFFECT OF PUROMYCIN AND CHLORAMPHENICOL ON RNA-DEPENDENT PROTEIN SYNTHESIS

Conditions	C^{14}-leucine (cpm incorporated)
Complete system	3650
+ puromycin (4×10^{-4} *M*)	44
+ chloramphenicol (3×10^{-4} *M*)	163
(Complete system minus f2 RNA	235)

The components given in *Methods* were incubated with C^{14}-leucine (1.5×10^7 cpm/μmole) at 35° for 60 min. Total volume was 0.25 ml, containing 50 μg of f2 RNA.

product, the other analysis of tryptic digests of the partially purified product. Of the counts incorporated into the acid-insoluble fraction, 45–55 per cent remains bound to the ribosomes when the ribosomes are removed by centrifugation, and the remainder is found in the 105,000 $\times$ *g* supernatant. It is this latter fraction only that we are studying. Analyses were done only when there was about a 40-fold stimulation over the background incorporation by the extracts so that the unstimulated product may essentially be neglected.

In the first experiment, the product was prepared by incubating C^{14}-leucine with the components described in *Methods*. The ribosomes were removed by centrifugation at 105,000 $\times$ *g* for 2 hr; 3 mg of f2 protein were added to an aliquot of the supernatant and this material was chromotographed on DEAE-cellulose in 3 *M* urea (in the absence of urea, f2 protein cannot be eluted) with a gradient of 0.1 to 1.0 *M* potassium phosphate, pH 7.4. Of the 3,000 acid-precipitable counts applied, 60 per cent was recovered of which 45 per cent was in the f2 protein peak.

The next two experiments take advantage of the reproducibility of the fingerprints of f2 protein. In the first of these experiments, the product was prepared by incubating the components described in *Methods* with 0.5 ml of preincubated S-30, 0.8 mg of f2 RNA, and C^{14}-arginine and lysine in a total volume of 2 ml at 35° for 90 min. After removal of the ribosomes by centrifugation at 105,000 $\times$ *g* for 2 hr, the supernatant contained 640,000 cpm in the hot acid-precipitable fraction. To an aliquot containing 140,000 cpm was added 3 mg of f2 protein and a large excess of C^{12}-arginine and lysine. The f2 protein was precipitated with ammonium sulfate at 20 per cent saturation, and the precipitate was washed with ammonium sulfate and suspended in water. By this procedure, 98 per cent of the acid-insoluble counts was precipitated with the carrier f2 protein. Although only 2 per cent of the product was in the supernatant, 3 mg of carrier f2 protein was added to this fraction, and it was carried through the procedure described below as a control for the adsorption of free C^{14}-amino acids by the carrier protein.

The two fractions were prepared for trypsin digestion by first precipitating and extracting with 5 per cent TCA at 90° for 15 min, followed by two TCA washes at room temperature, and then washing with ethanol-ether and ether. The dried product was subjected to performic acid oxidation by a modification of Hirs's procedure.[9] The oxidized protein was then digested for 3 hr at 25° in 1 ml of 0.05 *M* ammonium bicarbonate pH 7.9 containing 4 per cent trypsin by weight of substrate. After lyophilization to dryness, the digest was dissolved in 20 μl of water, an aliquot containing 105,000 cpm was applied to paper, and the peptides were separated by two-dimensional electrophoresis.

The electropherograms were radioautographed for five days and stained with ninhydrin to identify the peptides. Figure 3 shows the electropherogram of the

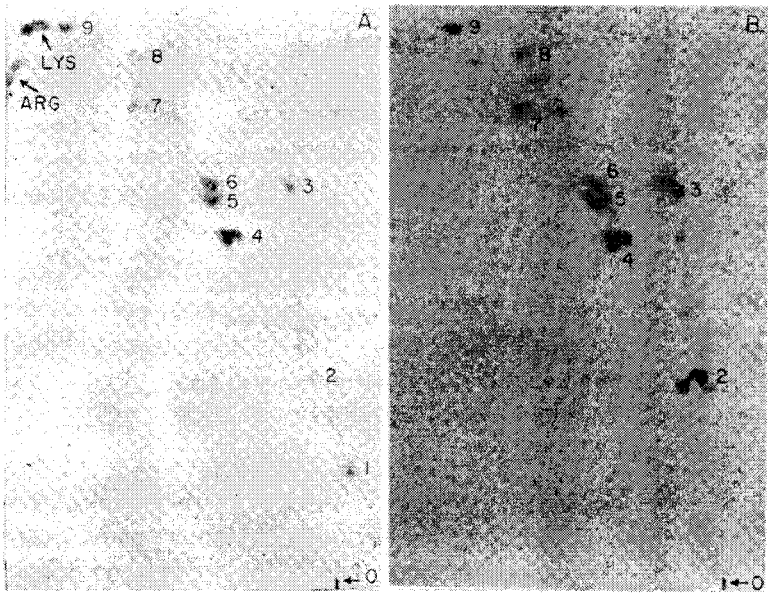

FIG. 3.—Electropherogram and radioautogram of the tryptic peptides from C^{14}-lysine- and arginine-labeled product and carrier f2 protein. *A* is the ninhydrin-stained paper, *B* is the radioautogram. "Arg" and "lys" represent markers; *O* indicates the origin.

ammonium sulfate precipitate. As the product was labeled with C^{14}-lysine and arginine, and since trypsin cleaves specifically at the carbonyl groups of lysine and arginine, we would expect that all the peptides would be labeled with the exception of the carboxy-terminal peptide. The nine peptides of f2 are marked 1 through 9. It may be noted that all peptides specifically identified with f2 except peptide number 1 are labeled. The congruence of the ninhydrin spots with the radioactive spots is immediately apparent even though the film is not superimposed here on the electropherogram. On the other hand, the electropherogram of the ammonium sulfate supernatant had the same ninhydrin peptides but the radioautogram was blank. This result was to be expected since, as already mentioned, 98 per cent of the TCA-precipitable counts was found in the original ammonium sulfate precipitate. This negative result does show, however, that the correspondence of ninhydrin and radioactive spots is not due to adsorption of C^{14}-lysine and arginine.

A similar control experiment was carried out using egg white lysozyme (Worthington) as carrier in place of f2 protein. In this instance, it was necessary to make the solution 60 per cent saturated with ammonium sulfate in order to precipitate the lysozyme. In other respects, the experiment proceeded as described above except that 50,000 cpm of the lysine and arginine-labeled product were applied to the paper. Although we obtained a whole new spectrum of ninhydrin spots in the electropherogram, the spots that appeared on the radioautogram (Fig. 4) corresponded with those of f2 protein and not with the lysozyme.

Note added in proof: Using C^{14}-lysine and -arginine, we have repeated the experiments described above with RNA isolated from *E. coli* K12 W6, starved of methionine (courtesy of L. Mandel and E. Borek), and with RNA isolated from

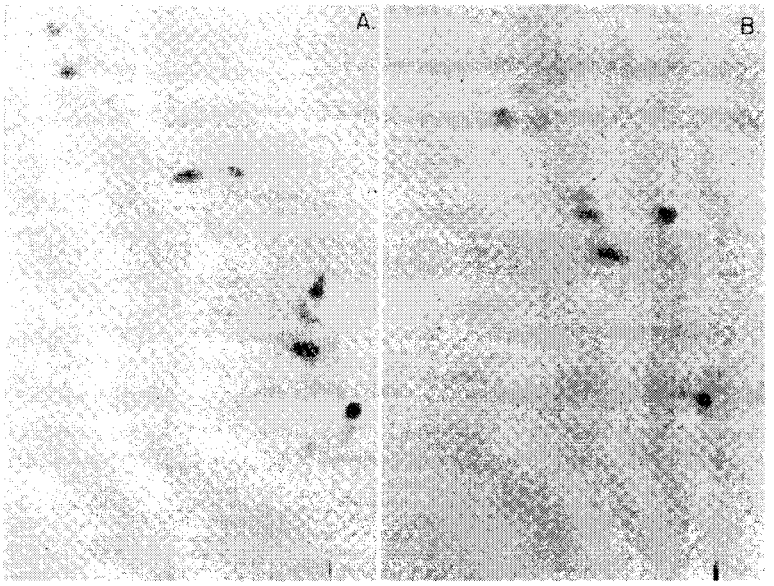

FIG. 4.—Electropherogram and radioautogram of the tryptic peptides from C^{14}-lysine- and arginine-labeled product and carrier lysozyme. *A* is the ninhydrin-stained paper, *B* is the radioautogram.

TMV (obtained from D. Caspar). Fingerprints of each product with f2 protein added as carrier showed no correspondence of radioactivity with the ninhydrin spots. In addition, the product prepared with f2 RNA was fingerprinted with TMV protein. Again there were no overlapping spots. We conclude that the product formed in the presence of f2 RNA is peculiar to the nucleic acid of the bacteriophage.

The next experiment used a second isotopic label to identify those f2 peptides containing leucine. Carrier protein labeled with H^3-leucine was to be mixed with the product labeled with C^{14}-leucine. If the product was identical to the phage protein, those peptides with H^3, i.e., containing leucine, would also have C^{14}, and those peptides without H^3, i.e., containing no leucine, would not have C^{14}.

A small amount of H^3-leucine-containing phage was made by growing f2 on a leucine-requiring strain of the host bacteria in the presence of high-specific-activity H^3-leucine. This phage was added to a large amount of pure, unlabeled phage, and protein was prepared. Fingerprinting of this material followed by elution and tritium counting showed that only peptides 2 and 4 contain leucine. C^{14}-leucine-labeled product was prepared as described above except for the change in C^{14}-amino acid. In this instance, the 105,000 $\times$ *g* supernatant contained 810,000 acid-insoluble cpm. An aliquot with 100,000 cpm (C^{14}) was added to the mixed carrier protein containing 200,000 cpm (H^3), and this material was precipitated and digested as previously described. In this experiment, only the ammonium sulfate precipitate was analyzed since, again, nearly all of the TCA precipitable counts were in this fraction. The ninhydrin-stained peptides were eluted from the electropherogram with hot water and an aliquot counted to determine the C^{14} and H^3 content. In Table 2, we can see that only peptides 2 and 4 contain substantial

TABLE 2

RADIOACTIVITY OF f2 PEPTIDES

Peptide	C^{14} (cpm)	H^3 (cpm)	C^{14}/H^3
1	200	300	...
2	1,100	2,510	0.44
3	355	0	...
4	2,960	10,600	0.28
5	0	80	...
6	40	0	...
7	0	24	...
8	232	0	...
Origin	4,400	7,000	0.63
Total	9,290	20,500	...
cpm applied	47,000	104,000	0.45
% recovered	20	20	...

radioactivity and each has both C^{14} and H^3. The ratio of the two isotopes in each peptide is approximately that of the original material applied to the paper. Only a few faint spots were seen on the radioautogram to indicate other C^{14}-containing material; these had no corresponding ninhydrin spots nor did they contain H^3. The product thus resembles f2 protein. It may be noted (Table 2) that only about 20 per cent of the counts applied to the paper were recovered. The balance of the counts could not be located even when elutions were performed with 6 *N* HCl, nor were they found in the solvents used for the electrophoresis. Since both product and carrier are lost proportionately, these unexplained losses do not alter the conclusion that f2 protein is being made.

Discussion.—The experiments presented leave little room for doubt that the synthesis of f2 coat protein can be mediated by f2 RNA when it is added to *E. coli* extracts. Since the isotopic labels appear primarily in the f2 peptides, we conclude that the coat protein forms a major component of the product that is released from the ribosomes. Preliminary evidence based on the fact that f2 RNA also stimulates the incorporation of histidine, an amino acid which is lacking in the coat protein, indicates that other protein is also being made. In this instance, more than half of the radioactive protein remains bound to the ribosomes.

An important question raised by these results is whether viral RNA acts directly as a template or through some intermediate such as its complementary strand. Although the hypothesis that RNA acts directly is more appealing, the latter possibility cannot, as yet, be excluded.

With the demonstration that viral RNA directs the synthesis of the coat protein in cell extracts, problems relating to viral replication can now be studied in an experimental system more easily controlled than the infected cell.

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